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Testing Protocol

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Supplemental Assay Method for Titration of Feline
Chlamydia psittaci in Cell Culture

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Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture

Table of Contents

1. Introduction
2. Materials
 - 2.1 Equipment/instrumentation
 - 2.2 Reagents/supplies
3. Preparation for the test
 - 3.1 Personnel qualifications/training
 - 3.2 Preparation of equipment/instrumentation
 - 3.3 Preparation of reagents/control procedures
 - 3.4 Preparation of the sample
4. Performance of the test
5. Interpretation of the test results
6. Report of test results
7. References
8. Summary of revisions
9. Appendices

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

1. Introduction

This Supplemental Assay Method (SAM) describes a titration method for assaying live cell culture-adapted feline *Chlamydia psittaci* vaccines for potency. The method uses a McCoy cell line as the test system. The presence or absence of the chlamydia agent is determined by staining inoculated cell cultures by an indirect fluorescent antibody (IFA) method using a monoclonal antibody (MAb) specific for feline *C. psittaci*.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Cabinet, laboratory biosafety level-2

2.1.2 Incubator, 36° ± 2°C, high humidity, 5% ± 1% CO₂ (Model 3336, Forma Scientific Inc.)

2.1.3 Incubator, 36° ± 2°C, aerobic (FA Model 2, Precision Scientific)

2.1.4 Water bath

2.1.5 Centrifuge, rotor, and rotor/microplate carriers (Model J-6B and Type JS-4.2 rotor, Beckman Coulter)

2.1.6 Microscope, ultraviolet (UV) light (Model BH2, Olympus America Inc.)

2.1.7 Pipettors, 20-µl, 200-µl, and tips

2.1.8 Multichannel pipettor, 50-300 µl x 12-channel

2.1.9 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

2.2.1 Chicken-embryo-adapted or cell-culture-adapted Chlamydia Reference, Cello Strain [available from the Center for Veterinary Biologics (CVB)]

2.2.2 Monospecific Antisera free of chlamydial neutralizing antibodies to nonchlamydia virus fractions present in multifraction Test Vaccines, e.g., feline panleukopenia virus, feline calicivirus, feline viral rhinotracheitis virus. [available from CVB]

2.2.3 *C. psittaci* MAb, FP 4E1FD7, feline specific

2.2.4 Anti-mouse fluorescein isothiocyanate conjugate (Anti-mouse FITC conjugate)

2.2.5 A McCoy cell line (McCoy B cell line, mouse fibroblast) free of extraneous agents as tested in accordance with the Code of Federal Regulations, Title 9 (9 CFR)

2.2.6 Minimum essential medium (MEM)

2.2.6.1 9.61 g MEM with Earles salts without bicarbonate

2.2.6.2 5.0 g lactalbumin hydrolysate or edamine

2.2.6.3 1.1 g sodium bicarbonate (NaHCO_3)

2.2.6.4 Q.S. to 1000 ml with deionized water (DW), and adjust the pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

2.2.6.5 Filter through a 0.22- μm filter.

2.2.6.6 Adjust the pH of the solution with 2N HCl to 7.1-7.2 following filtration.

2.2.6.7 Store at 2°- 7°C.

2.2.7 Growth medium for cell culture (Growth Medium)

2.2.7.1 Aseptically add to 900 ml MEM:

1. 100 ml gamma-irradiated fetal bovine serum (FBS)

2. Gentamicin sulfate, 50 $\mu\text{g/ml}$

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Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture

3. Store at 2°- 7°C.

2.2.8 Chlamydia Stock Solution

2.2.8.1 13.37 g Dulbecco's MEM with high glucose

2.2.8.2 3.7 g sodium bicarbonate

2.2.8.3 Q.S. to 1000 ml with DW; adjust the pH
to 6.8-6.9 with 2N HCl.

2.2.8.4 Sterilize through a 0.22-µm filter.

2.2.8.5 Aseptically add 50 µg/ml gentamicin
sulfate; adjust the final pH of the solution to
7.0-7.2 with 2N HCl.

2.2.8.6 Store at 2°- 7°C.

2.2.9 7.5% Sodium Bicarbonate

2.2.9.1 7.5 g sodium bicarbonate

2.2.9.2 Q.S. to 100 ml with DW.

2.2.9.3 Sterilize by autoclaving at 121°± 2°C,
15 psi for 30 ± 10 minutes.

2.2.9.4 Store at 2°- 7°C.

2.2.10 Maintenance Medium

2.2.10.1 Aseptically add:

1. 10.0 ml sterile tryptose phosphate
broth²⁴ (29.5 g/L in DW)

2. 2.0 ml 1M HEPES buffer

3. 4.0 ml gamma-irradiated FBS

4. 0.3 ml cycloheximide stock solution
(1000 µg/ml in DW) for a final concentration
of 3 µg/ml

5. Q.S. to 100 ml with Chlamydia Stock
Solution.

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Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture

6. Adjust pH to 7.0-7.2 with 7.5% Sodium Bicarbonate.

7. Store at 2°- 7°C.

2.2.11 Chlamydia Diluent

2.2.11.1 To prepare 100 ml Chlamydia Diluent, add aseptically:

1. 10.0 ml tryptose phosphate broth

2. 2.0 ml 1M HEPES buffer

3. Q.S. to 100 ml with Chlamydia Stock Solution.

4. Adjust pH to 7.0-7.2 with 7.5% Sodium Bicarbonate.

5. Store at 2°- 7°C.

2.2.12 Dulbecco's phosphate buffered saline (DPBS)

2.2.12.1 8.0 g sodium chloride (NaCl)

2.2.12.2 0.2 g potassium chloride (KCl)

2.2.12.3 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)

2.2.12.4 0.1 g magnesium chloride, hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)

2.2.12.5 Dissolve reagents in **Sections 2.2.12.1 through 2.2.12.4** with 900 ml DW.

2.2.12.6 Add 1.03 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4) to 10 ml DW, heat to 60° ! 2°C until dissolved, then add to **Section 2.2.12.5** with constant mixing.

2.2.12.7 Dissolve 0.1 g calcium chloride, anhydrous (CaCl_2) with 10 ml DW and add slowly to **Section 2.2.12.5** to avoid precipitation.

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

2.2.12.8 Q.S. to 1000 ml with DW; adjust the pH to 7.0-7.3 with 2N HCl.

2.2.12.9 Sterilize through a 0.22-µm filter.

2.2.12.10 Store at 2°- 7°C.

2.2.13 Cell culture plates, 96-well

2.2.14 Polystyrene tubes, 12 x 75-mm

2.2.15 Syringe, 1 ml tuberculin

2.2.16 Needles, 18-gauge x 1 1/2-inch

2.2.17 Acetone, 80%

2.2.17.1 80 ml acetone

2.2.17.2 20 ml DW

2.2.17.3 Store at room temperature.

2.2.18 Gauze. Cut into 20- x 15-cm, 4-layer pads; wrap in foil paper; sterilize by autoclaving at 121°± 2°C, 15 psi for 50 ± 10 minutes.

2.2.19 Labeling tape, 1/2-inch

3. Preparation for the test

3.1 Personnel qualifications/training

3.1.1 Personnel shall have experience in the preparation and maintenance of cell culture, as well as in the propagation and maintenance of chlamydial agents. Personnel shall be proficient with the quantitation techniques for chlamydia infectivity including IFA.

This method requires the propagation of *C. psittaci* agents which may be infectious for humans. All cultures and discarded materials should be considered potentially infective and handled in a manner consistent with safe laboratory practices and in accordance with the recommendations of the Centers for Disease Control and Prevention and the National Institutes of Health. All cultures, spent medium, and

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

contaminated materials should be disinfected or autoclaved before discarding. The use of appropriate Laboratory Biosafety Level-2 cabinets is recommended for these procedures.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of cell seeding and then on the day of cell inoculation, set a water bath at $36^{\circ} \pm 2^{\circ}\text{C}$ and the centrifuge temperature at $36^{\circ} \pm 2^{\circ}\text{C}$.

3.2.2 On the day of the IFA procedure, prepare a humidity chamber in the aerobic incubator by filling a pan in the bottom with DW.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of McCoy cell culture plates (McCoy Plates)

3.3.1.1 Prepare cells from healthy, confluent McCoy cells that are maintained by passing every 3 to 4 days. Using a multichannel pipettor, seed all wells of a 96-well cell culture plate 2 days prior to test initiation with 200 μl /well of cells suspended in growth media. The cell suspension should contain approximately $10^{5.39}$ to $10^{5.54}$ cells per ml. These become the McCoy Plates.

3.3.1.2 Incubate the McCoy Plates in a CO_2 incubator at $36^{\circ} \pm 2^{\circ}\text{C}$ for 2 days prior to inoculation. Only confluent cell monolayers should be used for the assay. (As illustrated in **Appendix I**. Avoid the use of outside wells if cell monolayers in those wells are not confluent.)

3.3.2 Preparation of the Chlamydia Reference Control

3.3.2.1 On the day of test initiation, rapidly thaw a vial of Chlamydia Reference in a $36^{\circ} \pm 2^{\circ}\text{C}$ water bath.

3.3.2.2 Dispense 1.8 ml Chlamydia Diluent into an appropriate number of 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the Center for Veterinary Biologics (CVB) Reagent Data Sheet and label (e.g., 7 tubes, labeled 10^{-1} through 10^{-7} , respectively).

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

3.3.2.3 With a 200- μ l pipettor, transfer 200 μ l of the Chlamydia Reference to the tube labeled 10^{-1} ; mix by vortexing.

3.3.2.4 Using a new pipette tip, transfer 200 μ l from the 10^{-1} -labeled tube (**Section 3.3.2.3**) to the 10^{-2} tube; mix by vortexing.

3.3.2.5 Repeat **Section 3.3.2.4** for each of the subsequent dilutions, transferring 200 μ l from the previous dilution tube to the next dilution tube until the dilution sequence is completed.

3.3.3 Preparation of the Working Chlamydia MAb

On the day the IFA procedure is conducted, dilute the Feline-specific *C. psittaci* MAb with DPBS as stated on the CVB Reagent Data Sheet.

3.3.4 Preparation of the Working Anti-mouse FITC Conjugate

On the day the IFA procedure is conducted, dilute the Anti-mouse FITC Conjugate with DPBS to its previously determined optimal working concentration.

3.4 Preparation of the sample

3.4.1 The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). On the day of inoculation, using a sterile 1.0-ml syringe and an 18-gauge x 1 1/2-inch needle, rehydrate a vial of the Test Vaccine by transferring 1.0 ml for 1-ml-dose vaccines, 0.5 ml for 1/2-ml-dose vaccines, etc., of the provided diluent into the vial containing the lyophilized Test Vaccine; mix by vortexing. Incubate for 15 ± 5 minutes at room temperature.

3.4.2 For multifraction feline *C. psittaci* vaccines, neutralize the nonchlamydia fractions with antiserum specific to each virus fraction.

3.4.2.1 Prepare dilutions of each neutralizing Monospecific Antiserum (nonchlamydia) according to the CVB Reagent Data Sheet or the manufacturer's instructions.

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

3.4.2.2 With a 200- μ l pipettor and a different tip, dispense 200 μ l of each Monospecific Antiserum into a 12 x 75-mm polystyrene tube, labeled 10^{-1} , and q.s. to 1.8 ml with Chlamydia Diluent. For example, to neutralize the 3 viral fractions of a feline chlamydia/FVR/FCV/FPV vaccine, dispense 200 μ l of each of the diluted FVR, FCV, and FPV antisera into the tube labeled 10^{-1} ; add 1.2 ml of Chlamydia Diluent to obtain a final volume of 1.8 ml.

3.4.2.3 Pipette 200 μ l of the reconstituted Test Vaccine to the labeled tube to yield a 10^{-1} dilution; mix by vortexing.

3.4.2.4 Incubate at room temperature for 30 ± 5 minutes.

3.4.3 For Test Vaccines containing only a feline chlamydia fraction, prepare the 10^{-1} dilution by adding 200 μ l of the Test Vaccine to 1.8 ml of Chlamydia Diluent in a 12 x 75-mm polystyrene tube, labeled 10^{-1} ; mix by vortexing.

3.4.4 Serial tenfold dilutions

Using the same method described for diluting the Chlamydia Reference Control in **Sections 3.3.2.2 through 3.3.2.5**, prepare an appropriate number of tenfold serial dilutions of the reconstituted Test Vaccine to bracket its expected endpoint specified in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production.

4. Performance of the test

4.1 On the day of test initiation, label the McCoy Plates, aseptically decant the Growth Medium, and blot the plate on a sterile gauze pad.

4.2 Inoculate 8 wells/dilution with 100 μ l/well of the Test Vaccine dilutions and the Chlamydia Reference Control dilutions (dilutions 10^{-3} through 10^{-7} for the example in **Section 3.3.2.2**). Change tips between each unique sample (e.g., each Test Vaccine and the Chlamydia Reference Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

the most concentrated within that series (e.g., 10^{-7} through 10^{-3}) (**Appendices I and II**).

4.3 Dispense 100 μ l of Chlamydia Diluent to each of 8 wells which serve as uninoculated cell culture controls (Cell Culture Controls) (**Appendices I and II**).

4.4 Seal each McCoy Plate by placing around the upper side of each plate sufficient 1/2-inch labeling tape to maintain the plate's lid in place.

4.5 Centrifuge the McCoy Plates at 539 x g (1500 rpm in a Beckman J-6B centrifuge, JS-4.2 Rotor, and Micro-Test Plate Carriers) at $36^{\circ} \pm 2^{\circ}\text{C}$ for 55 ± 5 minutes to aid adsorption and penetration of chlamydia into the McCoy cells.

4.6 After centrifugation, pipette 100 μ l/well of Maintenance Medium into the McCoy Plates including the Test Vaccine, Reference Control, and Cell Culture Controls.

4.7 Incubate the McCoy Plates at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO_2 incubator for 5 ± 1 days.

4.8 After incubation, decant the medium into an autoclavable waste container and pipette 200 μ l of DPBS into each well of the McCoy Plates.

4.9 Decant the DPBS in the same autoclavable waste container as in **Section 4.8**, and pipette 200 μ l of 80% acetone into each well of the McCoy Plates; incubate at room temperature for 15 ± 5 minutes.

4.10 Decant the 80% acetone in a suitable waste container, and gently blot the plate on paper towels. Allow the plates to air dry.

4.11 Dispense 90 ± 10 μ l of the Working Chlamydia MAb into each well of the McCoy Plates.

4.12 Incubate the McCoy Plates at $36^{\circ} \pm 2^{\circ}\text{C}$ in a high humidity aerobic incubator for 30 ± 5 minutes.

4.13 Decant the MAb; wash the cells twice by pipetting and decanting 200 μ l/well of DPBS. The first wash is decanted immediately after delivering the DPBS, whereas the second wash is allowed to remain for 10 ± 5 minutes before decanting. Decant and blot the plate.

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

4.14 Pipette $90 \pm 10 \mu\text{l}$ of the Working Anti-mouse FITC Conjugate into each well of the McCoy Plates.

4.15 Incubate the McCoy Plates in a high humidity aerobic incubator at $36^{\circ} \pm 2^{\circ}\text{C}$ for 30 ± 5 minutes.

4.16 Repeat **Section 4.13**.

4.17 Fill wells completely with DW, decant, and air dry.

4.18 Examine the plates at 200X using a UV-light microscope for typical intracytoplasmic, apple-green fluorescent inclusions which are characteristic of *C. psittaci* infection.

4.18.1 Wells containing 1 or more cells exhibiting *C. psittaci* fluorescent inclusions are considered positive for feline *C. psittaci*.

4.18.2 Results are recorded as the number of IFA-positive wells versus the total number of wells examined for each dilution of a Test Vaccine and the Chlamydia Reference Control.

4.19 Calculate the chlamydia endpoints of the Test Vaccine and the Chlamydia Reference Control using the Spearman-Kärber method as commonly modified by Finney. The titers are expressed as \log_{10} 50% fluorescent antibody infective dose (FAID₅₀) per ml or per dose.

Example:

10^{-2} dilution of Test Vaccine = 8/8 wells IFA positive
 10^{-3} dilution of Test Vaccine = 5/8 wells IFA positive
 10^{-4} dilution of Test Vaccine = 1/8 wells IFA positive
 10^{-5} dilution of Test Vaccine = 0/8 wells IFA positive

Test Vaccine titer = $(X - d/2 + [d \bullet S])$ where:

X = \log_{10} of dilution with all wells IFA positive(2)

d = \log_{10} of dilution factor (1)

S = sum of proportions of wells IFA positive for all dilutions tested:

$$\frac{8}{8} + \frac{5}{8} + \frac{1}{8} + \frac{0}{8} = \frac{14}{8} = 1.75$$

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

Test Vaccine titer = $(2 - 1/2) + (1 \bullet 1.75) = 3.25$

Adjust the titer to the recommended Test Vaccine dose as follows:

A. divide the **Test Vaccine Dose** by the **Inoculation Dose**

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this chlamydia test vaccine, the recommended dose is 1 ml)

Inoculation Dose = amount of diluted Test Vaccine added to each well of the Test Plate (for this chlamydia test vaccine, the inoculation dose is 0.1 ml)

$$\frac{1 \text{ ml dose}}{0.1 \text{ ml}} = 10$$

B. calculate \log_{10} of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

$$\text{Log of } 10 = 1.0$$

Test Vaccine Titer = $3.25 + 1.0 = 4.25$

Therefore the titer of the **Chlamydia Test Vaccine** is $10^{4.25}$ FAID₅₀/ml.

5. Interpretation of the test results

5.1 For a valid assay

5.1.1 The Cell Culture Controls shall not exhibit intracytoplasmic fluorescent inclusions, significant degradation of the cell sheet, CPE, or cloudy media that would indicate contamination.

5.1.2 The calculated FAID₅₀ titer of the Chlamydia Reference Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established by a minimum of 10 previously determined chlamydia titrations.

5.1.3 The lowest inoculated dilution of the Reference Control must induce specific IFA in 100% of the wells (8/8). If an endpoint is not reached (1 or more wells are IFA positive at the highest dilution), the titer is

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

expressed as "greater than or equal to" the calculated titer. If an endpoint is critical to testing, the highest (most dilute) must exhibit no IFA staining (0/8).

5.1.4 If the validity requirements are not met, then the assay is considered a **NO TEST** and can be retested without prejudice.

5.2 In a valid test, if the titer of the Test Vaccine is equal to or greater than the titer specified in the APHIS filed Outline of Production, the Test Vaccine is considered **SATISFACTORY**.

5.3 In a valid test, if the titer of the Test Vaccine is lower than the titer specified in the APHIS filed Outline of Production, the Test Vaccine shall be retested in accordance with the 9 CFR, Part 113.8(b).

6. Report of test results

Report results as FAID₅₀ per dose of the Test Vaccine.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.71, U.S. Government Printing Office, Washington, DC, 2004.

7.2 Cottral GE (Ed.). *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY, 1978, pg. 731.

7.3 Finney, DJ. *Statistical method in biological assay*, 3rd edition. Griffin, London, 1978, pp. 394-401.

7.4 Richmond JY, McKinney RW (Eds.). *Biosafety in microbiological and biomedical laboratories*. U.S. Department of Health and Human Services, U.S. Government Printing Office, Washington, DC, 1993, pg. 177.

7.5 Smith TF. *Chlamydia*. In *Diagnostic Procedures for viral, rickettsial and chlamydial infections*, 6th edition, NJ Schmidt and RW Emmons (Eds.). American Public Health Assoc, Washington, DC. 1989, pg. 1165-1192.

**Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture**

7.6 Spears, P and Storz, J. *Biotyping of Chlamydia psittaci based on inclusion morphology and response to diethylaminoethyl-dextran and cycloheximide*. Infect. Immun. 24:224-232, 1979.

7.7 Spears, P and Storz, J. *Chlamydia psittaci: Growth characteristics and enumeration of serotypes 1 and 2 in cultured cells*. J. Infect. Dis. 140:959-967, 1979.

7.8 Storz, J. *Chlamydia and chlamydia-induced diseases*. Charles C. Thomas Publishing Company, Springfield, IL., 1971, pg.358.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.2.6.3** The amount of sodium bicarbonate (NaHCO_3) has been changed from 2.2 g to 1.1 g.
- **4.17** Additional steps have been added for clarification.
- **4.19** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- **5.1.3** Recording the rate of positive reaction for validity requirements.
- The refrigeration temperatures have been changed from $4^\circ \pm 2^\circ\text{C}$ to $2^\circ - 7^\circ\text{C}$. This reflects the parameters established and monitored by the Rees system.
- "Test Serial" has been changed to "Test Vaccine" throughout the document.
- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.
- The footnotes have been deleted with any pertinent references now noted next to the individual items.

Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture

9. Appendices

Appendix I
Chlamydia Test Plate

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------------|---|---|---|---|---|---|---|---|----|----|----|
| A | | | | | | | | | | | | |
| B | 10^{-1} * | | → | → | → | → | → | → | → | CC | CC | |
| C | 10^{-2} | | → | → | → | → | → | → | → | CC | CC | |
| D | 10^{-3} | | → | → | → | → | → | → | → | CC | CC | |
| E | 10^{-4} | | → | → | → | → | → | → | → | CC | CC | |
| F | 10^{-5} | | → | → | → | → | → | → | → | | | |
| G | 10^{-6} | | → | → | → | → | → | → | → | | | |
| H | | | | | | | | | | | | |

CC = Cell Culture controls

* = Tenfold dilutions of Chlamydia Test Vaccine

Supplemental Assay Method for Titration of Feline *Chlamydia psittaci*
in Cell Culture

Appendix II
Chlamydia Reference Control Plate

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|--------------------|---|---|---|---|---|---|---|---|----|----|----|
| A | | | | | | | | | | | | |
| B | 10 ⁻³ # | | → | → | → | → | → | → | → | CC | CC | |
| C | 10 ⁻⁴ | | → | → | → | → | → | → | → | CC | CC | |
| D | 10 ⁻⁵ | | → | → | → | → | → | → | → | CC | CC | |
| E | 10 ⁻⁶ | | → | → | → | → | → | → | → | CC | CC | |
| F | 10 ⁻⁷ | | → | → | → | → | → | → | → | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

CC = Cell culture control

= Tenfold dilutions of Chlamydia Reference Control